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Distribution of the Urease Gene Cluster among and Urease Activities of Enterohemorrhagic *Escherichia coli* O157 Isolates from Humans

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Enterohemorrhagic *Escherichia coli* (EHEC) O157 strains belong to two closely related major groups, which are differentiated by their sorbitol fermentation phenotypes. Here we studied the conservation of urease genes and their expression in sorbitol-fermenting (SF) and non-SF EHEC O157 isolates. PCR targeting *ure* genes (*ureA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G*) demonstrated that each of these genes was present in 58 of 59 EHEC O157:H7 isolates. In contrast, none of 82 SF EHEC O157:NM (nonmotile) isolates contained any of the *ure* genes. Hence, the absence of the urease genes distinguishes SF EHEC O157:NM strains from EHEC O157:H7, but this absence demonstrates that the urease genes are not useful genetic targets for the detection of EHEC strains, because SF EHEC O157:NM strains are missed by such a strategy. When examined for urease activity on Christensen agar and in the API 20E system, only one O157:H7 strain displayed urease activity and produced elevated levels of ammonia, which was subsequently confirmed by ammonia electrode measurement. Because the *ure* genes were absent from each of nine strains of *E. coli* O55:H7, the proposed progenitor of EHEC O157, we hypothesize that EHEC O157:H7 diverged from the evolutionary pathway at an early stage and then acquired the O islands carrying the *ure* gene cluster.

The enzyme urease catalyzes the hydrolysis of urea to give ammonia and carbon dioxide, thereby providing an important nitrogen source for many bacterial species. In addition, urease can contribute to the virulence of several gram-negative bacteria and enhance their acid resistance (24). Ureolytic *Escherichia coli* strains are rarely found among clinical isolates. Recently, the completed genome sequence analysis of enterohemorrhagic *E. coli* (EHEC) O157:H7 strain EDL933 (27) and the Sakai O157:H7 outbreak strain (13) demonstrated two and one loci, respectively, consisting of seven urease genes (*ureD*, *-A*, *-B*, *-C*, *-E*, *-F*, and *-G*). Those genes are clustered in the same fashion as found in other members of the family of *Enterobacteriaceae*. In EHEC strain EDL933, the urease gene cluster is located within O islands OI 43 and OI 48 (27). Subsequent studies indicated that among various diarrheagenic *E. coli* strains from clinical sources, urease genes were associated with major EHEC groups O26, O111, and O157 (26) but were absent from diarrheagenic *E. coli* strains of other pathogroups, including enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and enteropathogenic *E. coli*. Among EHEC, particularly strains of serogroup O157, which cause diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome (HUS), are most important (31). Based on sorbitol fermentation and geographic distribution, EHEC O157 belong to two major groups (21). While non-sobitol-fermenting (non-SF) EHEC O157:H7 strains occur worldwide (31), SF EHEC O157:NM strains have emerged, so far, as important pathogens in Germany (10, 12, 21) and have caused two large outbreaks of HUS (2, 28). Multilocus enzyme electrophoresis, multilocus sequence typing, and determination of bacteriophage integration sites indicate that SF EHEC O157:NM strains are closely related to EHEC O157:H7 (21, 29). Both groups possess rfb_{O157} and *fliC* (encoding the H7 antigen), and most contain the stx_2 gene. Here we compared the two EHEC O157 groups for the presence of *ure* genes and urease production, using a large collection of EHEC O157:H7 and SF EHEC O157:NM strains isolated from patients in Germany.

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MATERIALS AND METHODS

Bacterial strains. Fifty-nine EHEC O157:H7 and 82 SF EHEC O157:NM strains were isolated between 1987 and 2003 from patients in Germany, Austria, and the Czech Republic. The EHEC O157:NM strains were from 72 patients with HUS and 10 patients with diarrhea. The EHEC O157:H7 strains were isolated from 31 patients with HUS and 28 patients with diarrhea. Between 1987 and 1994, such strains were detected by PCR screening of stools for *stx* genes, using primers MK1 and MK2 (19), and subsequent colony blot hybridization of PCR-positive stool cultures with P^{32}-labeled $stxA_1$ and $stxA_2$ probes (18). Between 1995 and 2003, EHEC O157 were isolated by immunomagnetic separation and culture on SMAC agar (20) followed by colony hybridization with digoxigenin-labeled *stx* probes (10) and agglutination of *stx*-positive colonies with anti-O157 serum (Oxoid, Unipath GmbH, Wesel, Germany). The nine *eae*-positive *E. coli* O55:H7 strains included strains TB156A and TB182A (5) and seven additional strains from our collection. The laboratory *E. coli* strain ATCC 25922 was included in the study as a negative control.

Urease activity. Cultures were examined for urease activity with Christensen urea agar plates and API 20E (BioMérieux, Marcy-l'Etoile, France) strips after overnight incubation at 37°C. The measurement of ammonia and the subsequent calculations were performed as described previously (7). Briefly, bacteria were cultured overnight at 37°C in 40 ml of urea broth supplemented with 1 μM $NiCl_2$ and dissolved into a 0.4 McFarland suspension. After a 20-min incubation at room temperature, 0.4 ml of 10 M NaOH was added, and the liberated ammonia was quantified with a 95–10 ammonia electrode (Orion Research, Cambridge, Mass.); ammonia concentrations were calculated by comparison with a standard curve. The procedure was performed three times per isolate to calculate the arithmetic mean and variances in measurement. Before use, the ammonia electrode was calibrated against a series of standard solutions. Differences between means of the ammonia concentration were calculated by performing the *t* test and Mann-Wilcoxon test. *P* values of <0.05 were considered to be statistically significant.

TABLE 1. PCR primers and conditions used in this study

Primer	Sequence	Target	PCR conditions			PCR product (bp)	Reference
			Denaturing	Annealing	Extension		
ureA-f	5′-GAC TCC AAG AGA AAA AGA CAA ACT A-3′	*ureA*	94°C, 60 s	63°C, 60 s	72°C, 40 s	271	This study
ureA-r	5′-CAG ATT ATC GGA TTA TGG ACG GTA-3′						
ureB-f	5′-GCT ACC TGC AGT ATT ATC GTT GAA-3′	*ureB*	94°C, 60 s	63°C, 60 s	72°C, 60 s	166	This study
ureB-r	5′-ACC CAT TAT ATC TCC GCG GAA ACC G-3′						
ureC-f	5′-TCT AAC GCC ACA ACC TGT AC-3′	*ureC*	94°C, 60 s	60°C, 60 s	72°C, 60 s	398	26
ureC-r	5′-GAG GAA GGC AGA ATA TTG GG-3′						
ureD-f	5′-CGT CAT CAT GTC GGT CTG CTC A-3′	*ureD*	94°C, 60 s	63°C, 60 s	72°C, 40 s	569	This study
ureD-r	5′-GCG TGG CTC CGG CGT AGT TTT-3′						
ureE-f	5′-GAG ACC CCG GCT CAG ACA ACT-3′	*ureE*	94°C, 60 s	63°C, 60 s	72°C, 60 s	400	This study
ureE-r	5′-CGTGATTATGGGCGTGCGACTT-3′						
ureF-f	5′-ACT GGA GTG GGC AGT GGA AGC-3′	*ureF*	94°C, 60 s	63°C, 60 s	72°C, 60 s	564	This study
ureF-r	5′-ACGGAATAATCGGGAATACTGGG-3′						
ureG-f	5′-GGT CCG GTC GGC TCA GGT AAA-3′	*ureG*	94°C, 60 s	63°C, 60 s	72°C, 60 s	518	This study
ureG-r	5′-GAT GTT TTG CAG ACC TTC ACC AC-3′						

PCR. PCRs for detecting the *ure* genes (*ureA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G*) were performed in a cycler from Biometra GmbH, Göttingen, Germany. PCR reagents were purchased from Peqlab Biotechnologie (Erlangen, Germany). The 50-μl PCR mixture consisted of 5 μl of bacterial suspension (ca. 10^4 bacteria), 5 μl of 10-fold-concentrated polymerase synthesis buffer Y containing 2.0 mM $MgCl_2$, 10 μl of Enhancer solution, 1 μl of 10 mM deoxynucleoside triphosphates, 30 pmol of each primer, and 1.25 U of *Taq* DNA polymerase. PCR primers, conditions, and target genes are shown in Table 1.

Southern blot hybridization. Genomic DNAs from three reference strains (*E. coli* O157:H7 strain EDL933, SF *E. coli* O157:NM strain 493/89, and *E. coli* O55:H7 strain TB182A) were digested with HindIII and EcoRI (New England Biolabs, Frankfurt, Germany) for hybridization with *ureA*, *-B*, *-C*, *-D*, *-F*, and *-G* probes; for hybridization with the *ureE* probe, digestion was performed with HindIII only. DNA fragments were separated on a 0.8% (wt/vol) agarose gel, transferred to a nylon membrane (Roti-Nylon plus; Carl Roth GmbH, Karlsruhe, Germany) by capillary blotting, and fixed by UV cross-linking (CL-1000 UV cross-linker; UVP, Inc., Upland, Calif.). The membrane was hybridized with the probes that were derived from *E. coli* O157:H7 strain EDL933 by PCR with primers listed in Table 1 and labeled with digoxigenin-11-dUTP by using a DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Stringent hybridization was achieved with the DIG DNA labeling and detection kit according to the manufacturer's instructions.

Sequence analysis. PCR products were purified (PCR purification kit; Qiagen, Hilden, Germany) and sequenced with an automated ABI Prism 3100 Avant Genetic Analyzer and the ABI Prism BigDye Terminator Ready Reaction Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Sequences were analyzed with the DNASIS program (Hitachi Software) and compared with sequences from the National Center for Biotechnology Information database.

RESULTS

Distribution of *ure* genes among EHEC O157 and *E. coli* O55:H7 isolates. Each of the seven *ure* genes was present in 58 of 59 EHEC O157:H7 strains isolated from patients with diarrhea or HUS (Table 2). In contrast, these genes were absent from each of 82 SF EHEC O157:NM strains and from each of nine *eae*-positive *E. coli* O55:H7 strains which did not contain *stx* genes.

Southern blot hybridization. To confirm the absence of the *ure* genes in SF EHEC O157:NM and *E. coli* O55:H7 strains, genomic DNAs from reference strains 493/89 and TB182A, respectively, were hybridized with digoxigenin-labeled *ureA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G* probes. *E. coli* O157:H7 strain EDL933 and *E. coli* strain ATCC 25922 were used as positive and negative controls, respectively. Southern blot analysis demonstrated that each of the seven *ure* probes hybridized to a single fragment of genomic DNA (of approximately 1.2 and 9 kb) from EDL933 but not to DNA from any of the other three strains.

Sequence analysis of the *ure* PCR amplicons. As shown in Table 3, the nucleotide sequences of the PCR products derived from the *ure* genes of six representative *E. coli* O157:H7 strains were 99 to 100% identical to each other and to those of the corresponding *ure* genes from strain EDL933 (GenBank accession number NC002655) and the Sakai strain (GenBank accession number AP002554).

Expression of urease activity and ammonia production. When grown on Christensen agar and tested with API 20E strips, only one of the 150 strains investigated demonstrated urease activity. This strain (strain 4413/95) is of serotype O157:H7 and contains all of the *ure* genes. After 24 h of culture in urea broth, ammonia production by strain 4413/95 was significantly higher than that by strains EDL933, SF EHEC O157:NM 819/02, and ATCC 25922, which were all negative

TABLE 2. *ure* genes and ureolytic activity in EHEC O157 and *E. coli* O55:H7 strains

Serotype	No. of isolates	No. PCR positive for *ure* genes (*ureA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G*)	No. positive for ureolytic activity by:	
			Christensen agar	API 20E
O157:H7	59	58	1[a]	1[a]
SF O157:NM	82	0	0	0
E. coli O55:H7	9	0	0	0

[a] The strain that tested positive with the API 20E is identical to the strain that tested positive on Christensen agar.

TABLE 3. Results of PCRs with primers complementary to *ure* genes and nucleotide sequence homology of the PCR products to *ure* genes of EHEC O157:H7 strain EDL933

Strain no./yr of isolation	Serotype	Sorbitol fermentation	PCR results (% nucleotide sequence homology to EDL 933)						
			ureA	*ureB*	*ureC*	*ureD*	*ureE*	*ureF*	*ureG*
4413/95	O157:H7	−	+ (100)	+ (99)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
HUS 1249	O157:H7	−	+ (100)	+ (100)	+ (99)	+ (100)	+ (100)	+ (99)	+ (100)
HC 3159	O157:H7	−	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (99)
H631/88	O157:H7	−	+ (100)	+ (99)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
H903-36/92	O157:H7	−	+ (100)	+ (99)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
1194	O157:H7	−	+ (100)	+ (99)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
5781/91	O157:H7	−	−	−	−	−	−	−	−
5869/96	O157:NM	+	−	−	−	−	−	−	−
3573/98	O157:NM	+	−	−	−	−	−	−	−
301/00	O157:NM	+	−	−	−	−	−	−	−
679/02	O157:NM	+	−	−	−	−	−	−	−
493/89	O157:NM	+	−	−	−	−	−	−	−
486/03	O55:H7	+	−	−	−	−	−	−	−
ATCC 25922		+	−	−	−	−	−	−	−

on Christensen agar (Fig. 1). Strains EDL933, 819/02, and ATCC 25922 produced comparably low levels of ammonia (Fig. 1).

DISCUSSION

Since their first identification (17), SF EHEC strains of serotype O157:NM have emerged as important causes of diarrhea, bloody diarrhea, and HUS in Germany (10, 12, 21, 23). During the last several years they have been identified as human pathogens in other European and non-European countries, including Austria, the Czech Republic, Finland, Hungary, the United Kingdom, and Australia (1, 3, 4, 12, 21, 22). SF Shiga toxin-producing *E. coli* (STEC) O157:NM infections occur most frequently during cold months and disproportionately affect children under 3 years of age (12, 21). SF EHEC O157:NM strains are characterized by a specific combination of their phenotypic and virulence features, which differentiates them from non-SF EHEC O157:H7. Specifically, SF STEC O157:NM strains can ferment sorbitol overnight and produce β-D-glucuronidase; are assigned to phage type 88 or 23; possess stx_2 as their sole *stx* gene (21); do not contain the tellurite resistance and adherence-conferring island (TAI) (30) but possess the complete EHEC factor-for-adherence (*efa1*) gene (15), which is only rudimentarily present in STEC O157:H7 (15); and also contain the *cdt* cluster, which is only rarely found in EHEC O157:H7 (16). Moreover, an *sfp* cluster encoding a putative adhesin has been identified on the large plasmid of SF EHEC O157:NM and has been shown to be characteristic of such strains (11). SF STEC O157:NM strains are nonmotile, but they regularly possess the *fliC* gene, encoding the H7 antigen (21). Monday et al. (25) have recently shown that the loss of motility in SF *E. coli* O157:NM strains is due to a 12-bp in-frame deletion in *flhC* that is required for transcriptional activation of genes involved in flagellum biosynthesis. By investigating the distribution of urease-encoding genes among EHEC O157 strains, we demonstrate in this study that EHEC O157:H7 and SF O157:NM differ remarkably from each other by the presence of the *ure* gene cluster.

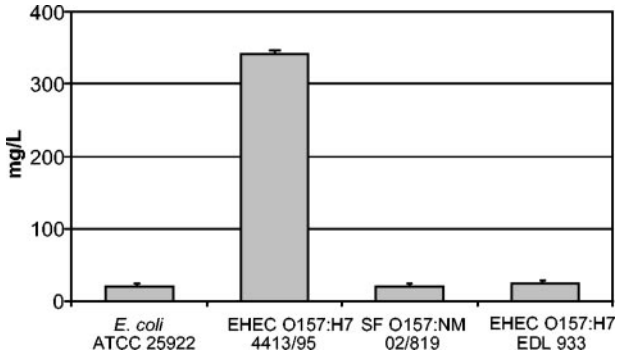

FIG. 1. Comparison of bacterial ammonia production after 24 h of culture in urea broth. Means ± standard deviations of ammonia measurements for *E. coli* strain ATCC 25922, EHEC O157:H7 strain 4413/95, SF O157:NM strain 493/89, and EHEC O157:H7 strain EDL933 are shown. The data presented are from three independent experiments.

The absence of *ure* genes from SF O157:NM strains, which has not been previously reported, has potential diagnostic implications. Nakano et al. (26) recently reported that the presence of *ureC* distinguishes EHEC of the major serogroups from diarrheagenic *E. coli* of other pathogroups and recommended this gene as a target to screen for EHEC. Our data clearly demonstrate that SF EHEC O157:NM strains would be missed by such an approach. On the other hand, PCR targeting *ureC* does provide a useful molecular tool for distinguishing SF EHEC O157:NM from EHEC O157:H7.

Multilocus enzyme electrophoresis demonstrates that SF EHEC O157:NM strains represent a distinct lineage within the EHEC O157:H7 clone complex (9, 34). In this evolutionary scenario, EHEC O157 evolved from an *E. coli* O55:H7 ancestor and then diverged into two groups, resulting in the O157:H7 clone, which spread worldwide, and the SF O157:NM clone, which is an increasing public health problem in Europe (9). Since the *ure* gene cluster is absent from both the *E. coli* O55:H7 strains and the SF EHEC O157:NM strains, it is possible that the EHEC O157:H7 strains have acquired the *ure* gene cluster later in their evolution by horizontal gene transfer.

Urease expression could modify internal and/or surrounding anion concentrations, enabling EHEC to survive acidic conditions and perhaps contributing to its low infectious dose and its

survival in the environment. In our study, only one of the 58 *ureC*-positive EHEC O157:H7 strains, which were isolated from patients with HUS or diarrhea, produced urease. The absence of urease production by the vast majority of EHEC O157 strains indicates that other acid tolerance mechanisms may be dominant in such strains. This low frequency of ureolytic activity in patients' isolates is similar to that found in *E. coli* O157:H7 strains isolated from patients in the United States (0.5%) (6) and Japan (7%) (35). In contrast, a substantially higher frequency of urease production (between 22 and 40%) has been reported in *E. coli* O157:H7 of bovine origin isolated in different countries (8, 32, 33). The reason for the low rate of urease expression among *E. coli* O157:H7 strains that are pathogenic for humans is unknown. Urease synthesis can be nitrogen regulated, urea inducible, or constitutive (24). Recent investigations demonstrated that the cloned EDL933 *ure* gene cluster was capable of synthesizing urease in an *E. coli* laboratory strain (14). However, when the *ure* gene cluster was transformed back into the native EDL933 background, the enzyme was not detectable, leading to speculation that an unidentified *trans*-acting factor(s) somehow down-regulates *ure* expression (14).

Because the *ure* gene cluster is regularly found in EHEC of serogroups O26, O111, and O157:H7, but not in other diarrheagenic *E. coli* strains (26), the question arises whether the presence of these genes contributes to the ability of EHEC to cause severe extraintestinal host injury. The fact that 88% of SF EHEC O157:NM strains in our study were isolated from HUS patients but none of these strains possessed urease-encoding genes suggests that the presence of these genes is not necessary for the ability of SF EHEC O157 to cause severe disease. However, if expressed in vivo, survival in the environment may be supported by the presence of *ure* genes in other EHEC strains.

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